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Effect of Lathyrism on Nucleic Acids and Subcellular Particles of the Experimental Granulation Tissue

By

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In earlier studies we have observed that in lathyrism (an intoxication caused by β-aminopropionitrile or aminoacetonitrile) the incorporation of amino acids in collagen was decreased (KULONEN, SALMI & JUVA 1960), the total concentration of nitrogenous substances in the tissue was decreased (VILJANTO, ISOMÄKI & KULONEN 1962), and the incorporation of amino acids into protein was retarded (SALMI & KULONEN 1962). It is also known (VILJANTO unpublished work) that in the granulation tissue the amount of RNA and the number of microsomes increase in parallel with the amount of collagen. This prompted a study of the effect of lathyrism on subcellular particles and the nucleic acids in experimental granuloma.

Experimental

Treatment of the animals. In the preliminary series 40 rats received daily each 40 mg aminoacetonitrile (AAN), beginning 10 days before the implantation of the cellulose sponges (VILJANTO & KULONEN 1962). The same number of rats served as controls. The food was restricted to about a half the normal consumption. The animals consequently became highly intoxicated, and several rats died; the values for their granulomas are not presented in detail, because the effects could have been non-specific. Ten days after the implantation the net dry weight of the granulomas was 56.7 ± 14.0 mg in the AAN-treated group and 119.6 ± 36.5 mg in the control group. The content of haemoglobin in the granulomas, which reflects the development of the capillaries, was 0.68 ± 0.47 mg/cm^3 in the AAN-group and 1.99 ± 0.42 mg/cm^3 in the control group. The increase of weight of the rats was zero in the AAN-group, but in the control group the increase was 0.77 g/rat/day.

The corresponding figures for the second main series are given in Table 1. The rats (45 experimental and 45 control rats) of the main series received less AAN (25 mg/rat daily) and more food (about three fourths of the normal consumption). The intoxication was now moderate, evidenced by the weight gain of the animals and by the formation of capillaries. No deaths occurred in this group.

The aminoacetonitrile was available as hydrosulphate (Abbot Laboratories, Chicago; gift of Dr. A. van den Hooff), which was dissolved in water; the solution (2 ml) was mixed with the usual food paste.

Before the implantation, 10–15 rats were kept in one cage; after implantation two rats lived in same cage with steel wire floor.

Implantation. The "Visella"-sponges (of viscose cellulose) were implanted as previously described (VILJANTO & KULONEN 1962; VILJANTO, ISOMÄKI & KULONEN 1962). The weight of the pieces in the main series was 72.6 ± 2.3 mg (air-dried) and the size 10 × 10 × 20 mm = 2 cm^3 (wet).

The rats were killed by ether 5 or 10 days after the implantation, and the sponges with their granulation tissue were extracted. The pieces were prepared free from the adjoining surface tissue, and the granulomas were stored frozen at −18°C.

Fractionation of the subcellular particles. Six granulomas were combined into a sample. Three samples were obtained and analysed independently.

The pieces were homogenized (in a Bühler homogenizer, 50000 r.p.m.) into 40 ml of 0.9% (w/v) NaCl-solution and allowed to flow through cheese-cloth to remove the coarse debris and parts of the sponge, which were washed with 20 ml of the same NaCl-solution. The homogenate was centrifuged at +4°C for 10 min. at 600 G. in an MSE refrigerated centrifuge. The sediment contained the fine debris and nuclei. It was washed twice with the NaCl-solution, and the washings were similarly centrifuged at 600 G. From the combined supernatants the mitochondrial fraction was obtained by 15 min. centrifugation at 5000 G. The sediment was washed five times with NaCl-solution, the washings were centrifuged at 6600 G. and the supernatants were combined. The microsomal fraction was obtained by centrifugation for 90 min. at 35000 G; it was washed once with NaCl-solution. The procedure was that of BOUCEK, NOBLE & WOESSNER (1959).

The sediments were washed into test tubes with 2 ml of NaCl-solution and stored frozen at −18°C.

Analyses. Haemoglobin was determined as cyanmethaemoglobin at the wave length of 5400 Å in the supernatant from separation of the microsomal fraction. To 1 ml of the supernatant were added 2 ml of the reagent containing 1% (w/v) potassium ferricyanide and 0.5% (w/v) sodium cyanide. For the values of molar extinction 11.5 and of the molecular weight 16520 were accepted. The result was calculated as mg of haemoglobin/cm^3 of granulation tissue.

The *dry weight* was determined after drying the pieces of granuloma for three days at +105°C. The net weight was obtained by subtraction.

For determining *nucleic acids* six granulomas (12 cm^3) were pooled (three samples). The pieces of tissue were homogenized into 2½ volumes of 0.9% NaCl-solution at the maximum speed of the Bühler-homogenizer. The homogenate was cooled to 0–1°C and an equal volume of 10% (w/v) cold trichloroacetic acid was added, mixed for 5 min. and centrifuged at 0°C for 15 min. at 10000 r.p.m. The sediment was washed with 50 ml of 96% (w/v) ethanol three times. The sediment was suspended in 30 ml of methanol, and the suspension was boiled under reflux for 1 hr.; this treatment was repeated once more. The sediment was further washed with 50 ml of 96% (w/v) ethanol and 50 ml of ether. The sediment thus obtained was extracted three times with 20 ml of 5% (w/v) trichloroacetic acid for 30 min. at +90°C. The extracts were combined and their volume made up to 60 ml with 5% trichloroacetic acid. The extraction procedure was a modification of that described by BIGGERS, LAWSON, LUCY & WEBB (1961).

The DNA was determined in the combined extracts by the diphenylamine reaction of BURTON (1956). For estimation of total nucleic acids the extract was adjusted to

0.75 N sulphuric acid and extracted with ether to remove the trichloroacetic acid. The extinction at 2625 Å was measured in the aqueous layer. The value of RNA was calculated by subtraction. As standard a preparation of "DNA ex herring sperm" (L. Light & Co. Ltd., Colnbrook, England) was used.

It was found that some DNA, and especially some RNA, remained in the organic washing fluids. The same source of error has been observed also by HALLINAN, FLECK & MUNRO (1963). The organic washings were evaporated to about 5 ml and made alkaline with 0.1 N sodium hydroxide. TCA was added to a concentration of 5%, and the solutions were heated at +90°C for 15 min. The nucleic acids were determined in this extract as described above, and the necessary correction was added to the results. This addition was largest for the RNA-values of the 10th day sample (0.17 mg/cm³ for AAN-samples and 0.53 mg/cm³ for normal samples).

The TCA-soluble nucleotides were also determined. The total ribonucleotide content of 10th day granulomas in the samples from AAN-treated animals was about half that of control animals.

Nitrogen and *hydroxyproline* were both determined after hydrolysis of the samples overnight at +100°C in closed tubes in 6 N HCl. Nitrogen was determined by a micro-Kjeldahl procedure and hydroxyproline by that of NEUMAN & LOGAN (1950).

Table 1.

Subcellular particles in granulation tissue*).

Description	5th day granuloma		10th day granuloma	
	control	AAN-treated	control	AAN-treated
Weight of the animals, g				
initial average	102	102	102	102
final average	120	116	121	117
increase rat/day	1.50	1.16	1.12	0.88
Hemoglobin in the granuloma, mg/cm³				
range	0.86–1.42	0.75–1.37	1.80–2.45	1.63–1.84
average	**1.19**	**0.98**	**2.10**	**1.75**
Nuclei and debris				
nitrogen, mg/cm³, range	0.110–0.252	0.077–0.144	0.146–0.314	0.073–0.185
nitrogen, mg/cm³, average	**0.184**	**0.112**	**0.219**	**0.140**
hydroxyproline, µg/cm³, range	1.05–2.40	1.10–1.70	2.80–13.95	2.50–6.80
hydroxyproline, µg/cm³, average	**1.87**	**1.35**	**7.38**	**4.83**
Mitochondrial fraction				
nitrogen, mg/cm³, range	0.086–0.155	0.032–0.085	0.161–0.220	0.096–0.193
nitrogen, mg/cm³, average	**0.117**	**0.060**	**0.187**	**0.132**
hydroxyproline, µg/cm³, range	0.80–1.40	0.60–1.35	1.40–2.35	1.00–1.70
hydroxyproline, µg/cm³, average	**1.12**	**0.97**	**1.97**	**1.33**
Microsomal fraction				
nitrogen, mg/cm³, range	0.106–0.156	0.087–0.138	0.259–0.348	0.183–0.221
nitrogen, mg/cm³, average	**0.139**	**0.112**	**0.315**	**0.198**
hydroxyproline, µg/cm³, range	1.15–1.25	0.90–1.30	1.65–2.80	1.50–2.35
hydroxyproline, µg/cm³, average	**1.18**	**1.15**	**2.35**	**1.90**

*) Animals of the main series only, 25 mg AAN daily. Three samples of six granulomas each were analyzed.

Results

Only the results of the second main series are given here in detail. Weight gain and vascularisation (as reflected by the haemoglobin content) are decreased in lathyritic animals, but the difference does not seem so large, as to make the granulation tissue non-viable (table 1). In the subcellular particles the effect of lathyrism is much more marked. The nuclear fraction may not be of great significance, because it contains much debris of undefined nature, but in the mitochondrial and microsomal fractions the decrease in nitrogen content seems genuine and is not due to contamination with small fragments of collagen. When the microsomal nitrogen is calculated on the basis of total DNA, there is no change due to lathyrism (table 2). The total amount of hydroxyproline bound to the subcellular fractions is little affected by the intoxication. On the contrary, the amount of hydroxyproline in the microsomal fraction, calculated on the basis of total DNA, total RNA or microsomal nitrogen (table 2), is increased. Caution is necessary, however, because in lathyrism the collagen may be present in such small fragments that they sediment at the same rate as microsomes.

Table 2.

Nucleic acids in granulation tissue*).

Description		5th day granuloma		10th day granuloma	
		control	AAN-treated*)	control	AAN-treated*)
DNA, mg/cm³	range	1.01–1.07	0.57–1.16	1.31–1.73	0.75–1.07
	average	**1.03**	**0.88**	**1.53**	**0.84**
RNA, mg/cm³	range	0.25–0.45	0.19–0.31	2.18–2.38	0.52–0.73
	average	**0.34**	**0.24**	**2.28**	**0.60**
RNA/DNA	range	0.25–0.42	0.25–0.33	1.38–1.66	0.68–0.74
	average	**0.33**	**0.28**	**1.51**	**0.70**
Microsomal N/tot. DNA, mg/mg	range	0.104–0.154	0.119–0.153	0.198–0.218	0.206–0.259
	average	**0.134**	**0.132**	**0.206**	**0.236**
Microsomal OP/tot. DNA, µg/mg	range	1.13–1.17	1.03–2.19	1.26–1.68	2.05–2.47
	average	**1.14**	**1.45**	**1.52**	**2.24**
Microsomal OP/tot. RNA, µg/mg	range	2.74–4.60	4.19–5.68	0.76–1.18	2.88–3.43
	average	**3.65**	**4.87**	**1.02**	**3.18**
Microsomal OP/N, µg/mg	range	7.37–10.84	9.42–11.36	6.37–8.05	8.20–10.63
	average	**8.75**	**10.37**	**7.37**	**9.54**

*) Animals of the main series only, 25 mg AAN daily. Three samples of six granulomas each were analyzed.

The amounts of both DNA and RNA are low in the granulomas of lathyritic animals; indeed the decrease is larger than the decrease in haemoglobin in the same granulomas. The effect is more marked in 10th day than in 5th day granulation tissue. From the ratios RNA/DNA it is evident that the synthesis of ribonucleic acid is especially retarded; the normal steep rise between the 5th and 10th days is moderate in the granulomas of lathyritic rats.

Discussion

More information on the RNA fractions is necessary for evaluating the mechanism and significance of defective nucleic acid synthesis. It is as yet not possible to correlate the disturbance in nucleic acid synthesis with the macromolecular maturation defect of collagen, which is the characteristic feature in lathyrism (NIKKARI & KULONEN 1962).

Summary

The content of nucleic acids (especially of RNA) was greatly decreased in the experimental granulation tissue of lathyritic rats. This diminution was more marked than the reduction observed in the weight gain of the rats or in the development of capillary circulation in the granulation tissue. The absolute amounts of the mitochondrial and microsomal nitrogen decreased also, but to a less extent. The total hydroxyproline was fairly high in the microsomal fractions of granulomas of lathyritic rats.

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